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(54) Title: ANTISENSE INHIBITORS OF THE HUMAN IMMUNODEFICIENCY VIRUS (57) Abstract Methods for modulating the expression of the HIV <i>tat</i> gene are disclosed comprising contacting <i>tat</i> RNA with oligonucleo- tide or oligonucleotide analog which can bind with at least a portion of the RNA. In accordance with the preferred embodiments, oligonucleotides or oligonucleotide analogs are designed to bind to portions of the <i>tat</i> RNA which are of significance to the ex- pression of the gene coding for said RNA. In accordance with a preferred embodiment, methods of treatment of human immu- nodeficiency virus are disclosed.		

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ANTISENSE INHIBITORS OF THE HUMAN IMMUNODEFICIENCY VIRUS

FIELD OF THE INVENTION

This invention relates to the field of therapeutics, particularly the treatment of infections of the human immunodeficiency virus (HIV). It relates to the design, synthesis and application of oligonucleotides and oligonucleotide analogs which inhibit the activity of the HIV and other retroviruses.

10 BACKGROUND OF THE INVENTION

This invention relates to materials and methods for modulating the activity of HIV RNA. The invention generally relates to the field of "antisense" compounds, compounds which are capable of specific hybridization with a nucleotide sequence of an RNA. In accordance with preferred embodiments, this invention is directed to methods for achieving therapeutic treatment of disease and regulating gene expression in experimental systems.

It is well known that most of the bodily states in mammals including infectious disease states, are effected by proteins. Such proteins, either acting directly or through their enzymatic functions, contribute in major proportion to many diseases in animals and man. Classical therapeutics has generally focused upon interactions with such proteins in efforts to moderate their disease causing or disease potentiating functions. Recently, however, attempts have been made to moderate the actual production of such proteins by interactions with molecules that direct their synthesis, intracellular RNA. By interfering with the production of

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proteins, it has been hoped to effect therapeutic results with maximum effect and minimal side effects. It is the general object of such therapeutic approaches to interfere with or otherwise modulate gene expression leading to undesired
5 protein formation.

One method for inhibiting specific gene expression which has been adopted to some degree is the "antisense" approach, where oligonucleotide analogs complimentary to a specific, target, messenger RNA, mRNA sequence are used. A
10 number of workers have reported such attempts. Pertinent reviews include C.A. Stein & J.S. Cohen, *Cancer Research*, vol. 48, pp. 2659-2668 (1988); J. Walder, *Genes & Development*, vol. 2, pp. 502-504 (1988); C.J. Marcus-Sekura, *Anal. Biochemistry*, vol. 172, 289-295 (1988); G. Zon, *Journal of Protein*
15 *Chemistry*, vol. 6, pp-131-145 (1987); G. Zon, *Pharmaceutical Research*, vol. 5, pp. 539-549 (1988); A. R. Van der Krol, J.N. Mol, & A.R. Stuitje, *BioTechniques*, vol. 6, pp. 958-973 (1988) and D.S. Loose-Mitchell, *TIPS*, vol. 9, pp. 45-47 (1988). Each of the foregoing provide background concerning general
20 antisense theory and prior techniques.

Prior attempts to inhibit HIV by various antisense approaches have been made by a number of researchers. Zamecnik and coworkers have used phosphodiester oligonucleotides targeted to the reverse transcriptase primer
25 site and to splice donor/acceptor sites P.C. Zamecnik, J. Goodchild, Y. Taguchi, P.S. Sarin, *Proc. Natl. Acad. Sci. USA* 83, 4143 (1986). Goodchild and coworkers have made phosphodiester compounds targeted to the initiation sites for translation, the cap site, the polyadenylation signal, the 5'
30 repeat region and a site between the gag and pol genes. J. Goodchild, S. Agrawal, M.P. Civeira, P.S. Sarin, D. Sun, P.C. Zamecnik, *Proc. Natl. Acad. Sci. U. S. A.* 85, 5507 (1988). In the Goodchild study, the greatest activity was achieved by targeting the polyadenylation signal. Agrawal and
35 coworkers have extended the studies of Goodchild by using chemically modified oligonucleotide analogs which were also targeted to the cap and splice donor/acceptor sites. S.

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- Agarwal, J. Goodchild, M.P. Civeira, A.H. Thornton, P.S. Sarin, P.C. Zamecnik, *Proc. Nat'l. Acad. Sci. USA* 85, 7079 (1988). A portion of one of these overlapped a portion of the HIV TAR region but was not found to have exemplary effect.
- 5 Neither was this oligonucleotide analog designed to interfere with the HIV TAR region. Agrawal and coworkers have used oligo-nucleotide analogs targeted to the splice donor/acceptor site inhibit HIV infection in early infected and chronically infected cells. S. Agrawal, T. Ikeuchi, D. Sun, P.S. Sarin,
- 10 A. Konopka, J. Maizel, *Proc. Natl. Acad. Sci. U. S. A.* 86, 7790 (1989).

- Sarin and coworkers have also used chemically modified oligonucleotide analogs targeted to the cap and splice donor/acceptor sites. P.S. Sarin, S. Agrawal, M.P.
- 15 Civeira, J. Goodchild, T. Ikeuchi, P.C. Zamecnik, *Proc. Natl. Acad. Sci. U. S. A.* 85, 7448 (1988). Zia and coworkers have also used an oligonucleotide analog targeted to a splice acceptor site to inhibit HIV. J.A. Zaia, J.J. Rossi, G.J. Murakawa, P.A. Spallone, D.A. Stephens, B.E. Kaplan, *J. Virol.*
- 20 62, 3914 (1988). Matsukura and coworkers have synthesized oligonucleotide analogs targeted to the initiation of translation of the rev gene mRNA. M. Matsukura, K. Shinozuka, G. Zon, et al, *Proc. Natl. Acad. Sci. USA* 84, 7706 (1987); R.L. Letsinger, G.R. Zhang, D.K. Sun, T. Ikeuchi, P.S. Sarin,
- 25 *Proc. Natl. Acad. Sci. U. S. A.* 86, 6553 (1989). Mori and coworkers have used a different oligonucleotide analog targeted to the same region as Matsukura. K. Mori, C. Boiziau, C. Cazenave, et al, *Nucleic Acids Res.* 17, 8207 (1989). Shibahara and coworkers have used oligonucleotide
- 30 analogs targeted to a splice acceptor site as well as the reverse transcriptase primer binding site. S. Shibahara, S. Mukai, H. Morisawa, H. Nakashima, S. Kobayashi, N. Yamamoto, *Nucl. Acids Res.* 17, 239 (1989). Letsinger and coworkers have synthesized and tested a oligonucleotide analogs with
- 35 conjugated cholesterol targeted to a splice site. K. Mori, C. Boiziau, C. Cazenave, et al, *Nucleic Acids Res.* 17, 8207 (1989). Stevenson and Iversen have conjugated polylysine to

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oligonucleotide analogs targeted to the splice donor and the 5'-end of the first exon of the tat gene. M. Stevenson, P.L. Iversen, *J. Gen. Virol.* 70, 2673 (1989). Buck and coworkers have recently described the use of phosphate-methylated DNA oligonucleotides targeted to HIV mRNA and DNA. H.M. Buck, L.H. Koole, M.H.P. van Gendersen, L. Smith, J.L.M.C. Green, S. Jurriaans and J. Goudsmit. *Science* 248, 208-212 (1990).

These prior attempts at targeting HIV have largely focused on the nature of the chemical modification used in the oligonucleotide analog. Although each of the above publications have reported some degree of success in inhibiting some function of the virus, a general therapeutic scheme to target HIV and other retroviruses has not been found. Accordingly, there has been and continues to be a long-felt need for the design of oligonucleotides and oligonucleotide analogs which are capable of effective, therapeutic antisense use.

This long-felt need has not been satisfied by prior work in the field of antisense oligonucleotide therapy for HIV and other retroviruses and viruses. Others have failed to identify target sites in which antisense oligonucleotides or oligonucleotide analogs are therapeutically effective at reasonable rates of application.

OBJECTS OF THE INVENTION

It is a principal object of the invention to provide therapies for human diseases, particularly the human immunodeficiency virus and other human retroviruses.

It is a further object of the invention to provide molecules, especially oligonucleotides and oligonucleotide analogs which perturb the structure of mRNA.

Yet another object of this invention is to modulate gene expression in cells.

A further object is to interfere with the secondary structure of RNAs through interaction of those structures with oligonucleotides or oligonucleotide analogs.

These and other objects of this invention will

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become apparent from a review of the instant specifications.

SUMMARY OF THE INVENTION

Prior attempts at antisense targeting to HIV have been focused on inhibition of the synthesis of some particular viral protein thought to be essential to the success of the infection. In the present invention, the same goal (inhibition of viral gene expression) is achieved, but greater, therapeutically significant activity is obtained by targeting particular sites on the HIV or other retrovirus RNA. In the present invention, target RNAs which have important biological function have been found to be the key target sites. It has been determined that targeting these RNA regions is a key to effective antisense therapy with oligonucleotides and oligonucleotide analogs.

It has now been discovered that compounds which specifically bind the tat RNA structure and interfere with tat trans-activation have activity as therapeutic agents for HIV infection. It is intended that all strains of HIV fall within the spirit and scope of this invention. While different strains of HIV may have different tat RNA sequences, this invention can be practiced on alternative strains of HIV by changing the sequence of the oligonucleotide or oligonucleotide analog to complement the structure of the alternative strain in accordance with the present teachings.

In accordance with the present invention, methods of modulating the expression of genes are provided. The targeted RNA, or cells containing it, is contacted with oligonucleotide or oligonucleotide analog which can bind with at least a portion of the RNA. The gene is generally one which is believed to give rise to a disease state in an organism and is typically a virus or, preferably, a retrovirus such as HIV, although other infectious organisms can be so attacked leading to therapeutic methods for treating diseased states by administering oligonucleotides or oligonucleotide analogs to animals suspected of having viral or retroviral infections.

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It has now been found that oligonucleotide or oligonucleotide analogs can be designed, especially for retroviruses such as HIV, which are effective in diminishing the infection. For HIV, a number of sequences have been found which are effective and persons of ordinary skill in the art will likely identify others. These sequences are the first, practically effective antisense sequences which have been shown to be effective in inhibiting the tat region of HIV and to lead to HIV therapeutics. Accordingly, this invention provides oligonucleotides and oligonucleotide analogs capable of binding with at least a portion of tat mRNA of HIV. Such oligonucleotides and analogs have been found corresponding to the nucleotide sequences G G C T C C A T T T C T T G C T C T C , CCATTTCTTGCTCTCCTCTGT, GCTATGTCGACACCCAATTC, CCGCCCCCTCGCCTCTTGCCG, CGGGTCCCCTCGGGATTGGG, and CACCTTCTTCTTCTATTCCT. It is preferred that the oligonucleotides and analogs have at least about 6 contiguous subunits of such sequences, with at least about 10 being preferred and at least about 15 being still more preferred. It is preferred for some embodiments that the oligonucleotide or oligonucleotide analog substantially correspond to a given sequence. By this is meant that the oligonucleotide or analog have every subunit of the sequence or an effective substitute. Thus, substitutions such as U for T and the like may be made in wild type oligonucleotides. Additionally, of course, other chemical modifications to form oligonucleotide analogs may be made without departing from the spirit of this invention.

The sequence portions may fall anywhere within the given sequences to likely have effect. Oligonucleotides and analogs, such as the preferred phosphorothioate analogs may be presented in a pharmaceutically acceptable carrier.

Methods of inhibiting gene expression, especially tat gene expression have also been discovered. Such inhibition may be employed for therapeutics, for diagnosis or for research. Thus, methods of treating an animal suspected of having a disease characterized by tat gene expression, especially AIDS, have been discovered comprising contacting

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the animal with oligonucleotides or oligonucleotide analogs in accordance with this invention.

It is preferred that the oligonucleotide or oligonucleotide analog be capable of binding with at least about six subunits of the RNA portion. It is more preferred that from eight to fifty units be capable of being bound, with from about 10 to about 20 subunits being even more preferred.

In accordance with preferred embodiments, the oligonucleotide or oligonucleotide analog is capable of forming a duplex structure with the portion of RNA. While the mechanism of the interaction is not known with certainty, it is possible that it may effect modulation of gene expression through a number of ways.

In accordance with preferred embodiments, the RNA portion which is interfered with comprises at least a part of the tat mRNA of HIV. The oligonucleotides and oligonucleotide analogs in accordance with this invention are, themselves believed to be novel. Thus, oligonucleotides which are capable of interacting with portions of tat RNA are comprehended. Thus, animals suspected of having the disease are contacted with oligonucleotide or oligonucleotide analog which can bind with the tat RNA. In particular, the present invention is believed to be effective in the treatment of HIV infections in mammals, especially man.

25 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a linear HIV-1 tat mRNA sequence.

Figure 2 depicts the activity of a series of oligonucleotides and oligonucleotide analogs in accordance with this invention in a cell culture assay for TAR/tat transactivation.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

It has been discovered to be possible to regulate the activity of HIV tat RNA in cells by introducing oligonucleotides or oligonucleotide analogs which bind to the tat mRNA. The oligonucleotides or oligonucleotide analogs

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interfere with the normal function of the mRNA and these methods can be used to treat diseases, particularly HIV.

In the context of this invention, the term oligonucleotide refers to a plurality of joined nucleotide units
5 formed from naturally-occurring bases and cyclofuranosyl groups joined by native phosphodiester bonds. This term effectively refers to naturally-occurring species or synthetic species formed from naturally-occurring subunits.

"Oligonucleotide analog," as that term is used in
10 connection with this invention, refers to moieties which function similarly to oligonucleotides but which have non naturally-occurring portions. Thus, oligonucleotide analogs may have altered sugar moieties or inter-sugar linkages. Exemplary among these are the phosphorothioate and other
15 sulfur containing species which are known for use in the art. They may also comprise altered base units or other modifications consistent with the spirit of this invention.

In accordance with certain preferred embodiments, at least some of the phosphodiester bonds of the
20 oligonucleotide have been substituted with a structure which functions to enhance the ability of the compositions to penetrate into the region of cells where the RNA whose activity is to be modulated is located. It is preferred that such linkages be sulfur-containing. It is presently preferred
25 that such substitutions comprise phosphorothioate bonds. Others such as alkyl phosphothioate bonds, N-alkyl phosphoramidates, phosphorodithioates, alkyl phosphonates, and short chain alkyl or cycloalkyl structures may also be useful. In accordance with other preferred embodiments, the
30 phosphodiester bonds are substituted with structures which are, at once, substantially non-ionic and non-chiral. Persons of ordinary skill in the art will be able to select other linkages for use in the practice of the invention.

It is generally preferred for use in some
35 embodiments of this invention that the 2' position of the linking sugar moieties in at least some of the subunits of the oligonucleotides or oligonucleotide analogs be substituted.

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Thus, 2' substituents such as OH, SH, F, OCH₃, OCN, OCH_nCH₃ where n is from 1 to about 20 and other substituents having similar properties may be useful in some embodiments.

Oligonucleotide analogs may also include species
5 which include at least some modified base forms. Thus, purines and pyrimidines other than those normally found in nature may be so employed. Similarly, modifications on the cyclofuranose portions of the nucleotide subunits may also occur as long as the essential tenets of this invention are
10 adhered to.

Such analogs are best described as being functionally interchangeable with natural oligonucleotides (or synthesized oligonucleotides along natural lines), but which have one or more differences from natural structure. All such
15 analogs are comprehended by this invention so long as they function effectively to bind to selected portions of tat RNA.

The oligonucleotides and oligonucleotide analogs in accordance with this invention preferably comprise from about 3 to about 100 subunits. It is preferred that such
20 oligonucleotides and analogs comprise at least about 6 subunits with from about 8 to about 50 subunits being more preferred, and still more preferred to have from about 10 to about 20 subunits. As will be appreciated, a subunit is a base and sugar combination suitably bound to adjacent subunits
25 through phosphodiester or other bonds.

The oligonucleotides and oligonucleotide analogs of this invention can be used in diagnostics, therapeutics and as research reagents and kits. For therapeutic use, the oligonucleotide or oligonucleotide analog is administered to
30 an animal, especially a human, such as are suffering from a virus or retrovirus infection such as AIDS.

It is generally preferred to apply the therapeutic agents in accordance with this invention internally such as orally, intravenously or intramuscularly. Other forms of
35 administration, such as transdermally, topically or intralesionally may also be useful. Inclusion in suppositories may also be useful. Use of the oligonucleotides

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and oligonucleotide analogs of this invention in prophylaxis is also likely to be useful. Use of pharmacologically acceptable carriers is also preferred for some embodiments.

In accordance with the present invention, it will be understood that the term "to bind" as it refers to the interaction between an oligonucleotide or oligonucleotide analog and an RNA portion or subportion may have any of several, related meanings. Thus, the present invention comprehends binding of an oligonucleotide or analog with at least one portion of tat RNA. It will be understood that the oligonucleotide or analog will bind with at least one portion of the RNA portion in a Watson-Crick fashion so as to form, locally, a heteroduplex between the RNA portion and the oligonucleotide or analog. This heteroduplex formation is believed to result in alteration of the structure or function of the RNA portion. The exact mechanism and the result of this effect is not known with certainty, yet it is believed that the normal structure or function of the RNA portion is gradually replaced by the binding of the oligonucleotide with one or more portions of the RNA. Since the electronic and steric factors which attend the new heteroduplex are different from those of the natural-occurring RNA portion, the effectiveness and nature of the function to generate protein from the RNA is interfered with. The resulting formation of defective or missing protein manifests itself overall as a modulation in the expression of the gene coding for the RNA.

In short, any interaction or binding of oligonucleotide or oligonucleotide analog with tat RNA is believed to have the potential for interference with RNA function and, hence, for modulation the expression of the gene from which the RNA derives. Tat RNA targets have been found which exhibit overall diminution of activity of HIV when oligonucleotides or oligonucleotide analogs corresponding to portions of the target are administered to infected cells.

While a wide variety of oligonucleotides and oligonucleotide analogs are believed to be useful in practice of the present invention, it has been found to be preferred

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to design such oligonucleotides and analogs so as to bind with at least about six subunits of a portion of RNA. In accordance with other preferred embodiments, oligonucleotides which combine with from about six to about 30 and even more preferably with about 10 to about 20 subunits are preferred. As discussed above, it is presently believed that the tat RNA of HIV comprises an excellent target for employment of the present invention. Accordingly, preparation of oligonucleotide or oligonucleotide analog for binding with one or more portions of the tat RNA region of HIV are preferred.

Therapeutics are particular objects of the present invention. Thus, presenting oligonucleotides and oligonucleotide analogs in accordance with the present invention in pharmaceutically acceptable carriers may be highly useful. This is especially true for treatment of the disease AIDS.

Overall, it is preferred to administer to patients suspected of suffering from the foregoing disease states with amounts of oligonucleotide or analog, in either native form or suspended in a carrier medium in amounts and upon treatment schedules which are effective to reduce the symptomology of that disease. It is within the scale of a person's skill in the art to determine optimum dosages and treatment schedules for such treatment regimens.

An elaborate set of control elements in the HIV genome determine whether the virus replicates or remains dormant. Of the nine genes identified in the HIV genome, only three are from the core and envelope. W.A. Haseltine, F. Wong-Staal, *Scientific American* October, 52 (1988). The other six genes are involved in regulation of the production of viral proteins. Regulatory genes work by encoding a protein that interacts with a responsive element somewhere else on the viral genome. The major regulatory gene responsible for initiating the burst of replication is the tat (trans-activator) gene. Figure 1 is a sequence for the cDNA of the tat region in HIV. The product of the tat gene, tat protein, works by interaction with a short sequence element known as

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TAR (trans-acting responsive element). The TAR sequence is encoded in the viral long terminal repeats (LTR's), and therefore is included in the mRNA from every HIV gene.

Expression of the *tat* protein results in increased
5 expression of other HIV genes up to 1,000 fold, including the *tat* gene itself. Because of this autoregulatory positive feedback, and the fact that the TAR sequence is included in the mRNA from every HIV transcript, a immense amount of viral gene expression is triggered when the *tat* gene is activated.
10 The interaction between the *tat* gene and the TAR element is therefore crucial to the life cycle of the HIV, and specific disruption of this interaction is likely to interrupt the propagation of the virus.

The mechanism of trans-activation of TAR-containing
15 genes by the *tat* protein has recently been studied intensely. Philip, A. Sharp, Robert, A. Marciniak, Cell 59, 229 (1989). Although much remains to be learned, two important points have become clear; that *tat* increases the expression of TAR-containing genes by increasing both the amount of viral mRNA
20 and the efficiency of its translation, and that TAR functions as an RNA structure, rather than a DNA structure.

The unusual conclusion that *tat* increases the transcription of TAR-containing genes, but does so by interacting with the TAR element in RNA was derived from a
25 number of observations. Philip, A. Sharp, Robert, A. Marciniak, Cell 59, 229 (1989). In order to achieve trans-activation, the TAR element must be located immediately downstream from the site of initiation of transcription. Moreover, TAR is orientation dependent; if inserted in the inverse orientation,
30 it fails to function.

Some of the strongest evidence that *tat* interacts with TAR as an RNA structure has come from mutagenesis experiments. Efforts to study the TAR element were stimulated by the observation that the *tat* protein from HIV-1 was capable
35 of trans-activating vectors containing the TAR region of HIV-2, a different strain of virus, even though there is very little primary sequence homology in the TAR region between the

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two strains. S. Feng, E.C. Holland, *Nature* 334, 165 (1988). However, examination of the TAR sequence from HIV-1 and HIV-2 with computer programs that predict RNA secondary revealed the potential of RNA stem-loop structures, with a single stem-loop in the TAR region of HIV-1 and three stem-loop structures in HIV-2. Although the compositions and lengths of the stems were divergent, all four loops contained the pentanucleotide CUGGG as shown in Figures 1A and 1B. Mutagenesis experiments revealed that each of the nucleotides present in the loop are absolutely essential for trans-activation by tat, but that base substitutions in the stem were tolerated to some extent so long as the stem structure was maintained. S. Feng, E.C. Holland, *Nature* 334, 165 (1988).

Further evidence for the TAR structure functioning as RNA was obtained from experiments in which the sequences flanking the stem-loop structure were altered creating competing secondary structures in the RNA that were more stable than the natural TAR stem-loop. B. Berkhout, *Cell* 59, 273 (1989). This was accomplished by introducing additional sequences into the TAR-containing RNA that were antisense to the 5' side of the stem-loop structure. Trans-activation of the modified TAR structure was lost, suggesting that the TAR sequences alone are not sufficient for trans-activation, but that these sequences must fold up in the proper secondary structure to be active. It also suggests that antisense sequences to the TAR stem-loop are capable of disrupting the natural RNA structure.

Direct biochemical evidence for TAR stem-loop structure has also been obtained. The TAR RNA has been enzymatically synthesized *in vitro* and probed with enzymes selectively cleave single stranded regions of RNA, but not duplex structures. The results of the enzyme cleavage patterns were consistent with the computer predicted RNA secondary structure. B. Berkhout, *Cell* 59, 273 (1989).

In summary, there is strong and direct evidence from a number of studies that the HIV tat protein is responsible for triggering an enormous amount of viral gene expression,

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that this occurs by interaction with the TAR sequence which is incorporated into every HIV mRNA transcript, that the HIV TAR sequence functions as an RNA structure and that the correct TAR RNA structure is essential for tat transactivation.

TAR and tat function has been studied by removing the genes from the HIV genome and studying them in cell lines in isolation. Vectors have been constructed to study the interactions between the tat protein and TAR element. The tat gene is expressed under the SV40 promoter. The TAR region is expressed from a separate plasmid fused to an easily assayed reporter gene, the placental alkaline phosphatase gene (PAP). P. Henthorn, P. Zervos, M. Raducha, H. Harris, T. Kadesch, *Proc. Natl. Acad. Sci. USA* 85, 6342 (1988). Enzymatic activity in cell culture models has been shown to be dependent upon both the presence of the essential elements of the TAR region and the presence of the tat protein. P. Sharp, R. Marciniak, *Cell* 59, 229 (1989); S. Feng, E.C. Holland, *Nature* 334, 165 (1988); Michael, F. Laspias, Andrew, P. Rice, Michael, B. Mathews, *Cell* 59, 283 (1989); J.A. Garcia, D. Harrich, E. Soultanakis, F. Wu, R. Mitsuyasu, R.B. Gaynor, *EMBO J.* 8, 765 (1989); and B. Berkhout, *Cell* 59, 273 (1989). In essence, the vector system reconstitutes the events of tat-mediated TAR transactivation in which occurs in HIV infected cells.

TAT/TAR trans activation can be conveniently assayed by placing the human placental alkaline phosphatase gene (PAP) under the regulatory control of the HIV-1 LTR sequences, which contain enhancer, promoter, and tar elements. A plasmid containing the HIV-1 LTR, pHIVCAT-0 (S. Feng, E.C. Holland, *Nature* 334, 165 (1988)), contains HIV U3 in its entirety and R up through position +78 (a HindIII site). Digestion of this plasmid with a combination of HindIII and AatII releases the CAT cassette along with the SV40 sequences responsible for the processing of the RNA. A second plasmid, pSV2Apap, contains the PAP cassette with eukaryotic processing signals, under the transcriptional control of an SV40 promoter. P. Henthorn, P.

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Zervos, M. Raducha, H. Harris, T. Kadesch, *Proc. Natl. Acad. Sci. USA* 85, 6342 (1988). The PAP cassette and processing sequences were released from the plasmid by digestion with HindIII and AatII. A new plasmid, pHIVPAP, was created by
5 ligating the HindIII/AatII fragment containing the HIV-1 LTR and vector sequences from pHIVCAT-0, to the HindIII/AatII PAP cassette from pSV2Apap.

To test the activity of oligonucleotide analogs, pcDEBtat and pHIVPAP were co-transfected into HeLa cells by
10 calcium/phosphate precipitation. The effects of the selected oligonucleotide analogs was determined as follows. HeLa cells were split 1:8 into 6-well dishes the day prior to the transfections. For each dish, 1ug of pHIVPAP and 12ug of pcDEBtat were precipitated in 500 μ l of HBS and 32 μ l of 2.5
15 M CaCl_2 . The CaPO_4 precipitate was divided evenly between the 6 wells. Oligonucleotides or oligonucleotide analogs were preprecipitated in the same manner and added to the cells at the concentrations indicated in the figures. The precipitate was allowed to sit on the cells for 20 minutes then complete media
20 was added and the cells were incubated for an additional 4 hours. The cells were then shocked with 10% glycerol in HBS.

After 48 hours, cells were harvested and protein and PAP assays performed as described by Henthorn et al. P. Henthorn, P. Zervos, M. Raducha, H. Harris, T. Kadesch, *Proc.*
25 *Natl. Acad. Sci. USA* 85, 6342 (1988) with the following modifications. The cells were harvested in 0.5 ml of TBS, of which 0.1 mls were used for use in the protein assay. The remaining 0.4 mls of cell suspension was pelleted then resuspended in 50 μ l TBS. Endogenous phosphatases were
30 inactivated by heating the cells at 65°C for 30 min. The heat stable human placental alkaline phosphatase activity was assayed by the addition of PNPP (0.5 ml, 5 mM PNPP) to the cell suspension, which was then incubated at 37°C. Activity was determined at 30 minute intervals using 150 μ l aliquots
35 of the reaction mixture and measuring absorbance at 405 nm with a Titertek Multiscan MCC\340 ELISA plate reader. The PAP activity was normalized to the total protein in each well

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as determined by Bio-Rad protein assay, in which 1\5 of the harvested cells in TBS(0.1 μ l) were added to 30 μ l of Bio-Rad Protein Reagent, then incubated for 10 minutes at room temperature, followed by measurement of absorbance at 595 nm using the Titertek plate reader.

Cells were treated with the following oligonucleotide analogs having phosphorothioate backbones:

#	5'	3'
461	GGCTCCATTTCCTTGCTCTCC	
10 462	CATTTCTTGCTCTCCTCTGT	
463	GCTATGTCGACACCCAATTC	
464	CCGCCCCCTCGCCTCTTGCCG	
465	CGGGTCCCCTCGGGATTGGG	
466	CACCTTCTTCTTCTATTCT	
15 Control	TGGCATCGAT GCTCA	

The data are displayed graphically in Figure 2. Significant diminution in PAP activity, which is a direct measure of gene expression from the HIV LTR, was exhibited by the phosphorothioate oligonucleotide analogs bearing code numbers 461, 462, 463, 464 and 465. Oligonucleotide 466 and the control oligonucleotide, which was not designed to be complementary to the tat mRNA did not exhibit significant activities in this assay. The apparent increase in PAP activity at the 1 μ M dose for the control oligonucleotide and compound number 466 probably resulted from a carrier effect in which the oligonucleotides facilitated uptake of the plasmids at the time of transfection. This effect may have been present in experiments with the active oligonucleotides, but was masked by the specific inhibitory activities of these compounds.

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WHAT IS CLAIMED IS:

1. An oligonucleotide or oligonucleotide analog comprising at least 6 contiguous subunits corresponding to any of the nucleotide sequences:

5 5' 3'
 GGCTCCATTTCTTGCTCTCC
 CATTTCTTGCTCTCCTCTGT
 GCTATGTGTCGACACCCAATTC
 CCGCCCCCTCGCCTCTTGCCG
10 CGGGTCCCCCTCGGGATTGGG
 CACCTTCTTCTTCTATTCT

2. The oligonucleotide or oligonucleotide analog of claim 1 comprising at least about 10 contiguous subunits.

3. The oligonucleotide or oligonucleotide analog
15 of claim 1 comprising at least about 15 contiguous subunits.

4. The oligonucleotide or oligonucleotide analog of claim 1 substantially corresponding to one of said sequences.

5. The oligonucleotide or oligonucleotide analog
20 of claim 1 in a pharmaceutically acceptable carrier.

6. An oligonucleotide or oligonucleotide analog capable of binding with at least a portion of tat mRNA of HIV.

7. The oligonucleotide or oligonucleotide analog of claim 6 comprising at least about 6 subunits.

25 8. The oligonucleotide or oligonucleotide analog of claim 6 comprising at least about 15 subunits.

9. The oligonucleotide or oligonucleotide analog of claim 6 comprising from about 8 to about 50 subunits.

30 10. The oligonucleotide or oligonucleotide analog of claim 6 comprising from about 10 to about 20 subunits.

11. The oligonucleotide or oligonucleotide analog of claim 6 capable of forming a heteroduplex with said portion.

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12. A method for treating an animal suspected of having a disease characterized by expression of the HIV tat gene comprising contacting said animal with an oligonucleotide or oligonucleotide analog comprising at least 6 contiguous subunits corresponding to any of the nucleotide sequences:

5' 3'

GGCTCCATTCTTGCTCTCC

CATTCTTGCTCTCCTCTGT

GCTATGTCGACACCCAATTC

10 CCGCCCCCTCGCCTCTTGCCG

CGGGTCCCCTCGGGATTGGG

CACCTTCTTCTTCTATTCCT

13. The method of claim 12 wherein said
oligonucleotide or oligonucleotide analog comprises at least
15 about 10 contiguous subunits.

14. The method of claim 12 wherein said oligonucleotide or oligonucleotide analog comprises at least about 15 contiguous subunits.

15. The method of claim 12 wherein said
20 oligonucleotide or oligonucleotide analog substantially
corresponds to one of said sequences.

16. The method of claim 12 wherein said oligonucleotide or oligonucleotide analog is in a pharmaceutically acceptable carrier.

25 17. A method for treating an animal suspected of having a disease characterized by expression of the HIV tat gene comprising contacting said animal with an oligonucleotide or oligonucleotide analog capable of binding with at least a portion of tat mRNA of HIV.

30 18. The method of claim 17 wherein said
oligonucleotide or oligonucleotide analog comprises at least
about 6 subunits.

19. The method of claim 17 wherein said oligonucleotide or oligonucleotide analog comprises at least
35 about 15 subunits.

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20. The method of claim 17 wherein said oligonucleotide or oligonucleotide analog comprises from about 8 to about 50 subunits.

21. The method of claim 17 wherein said oligonucleotide or oligonucleotide analog comprises from about 10 to about 20 subunits.

22. The method of claim 17 wherein said oligonucleotide or oligonucleotide analog is capable of forming a heteroduplex with said portion.

23. The method of claim 17 wherein said oligonucleotide or oligonucleotide analog is in a pharmaceutically acceptable carrier.

24. A method for treating an animal suspected of having a HIV infection comprising contacting said animal with an oligonucleotide or oligonucleotide analog capable of binding with at least a portion of tat mRNA of HIV.

25. The method of claim 24 wherein said animal is human.

26. A method for modulating expression of the HIV tat gene comprising contacting cells or an animal infected with HIV with an oligonucleotide or oligonucleotide analog comprising at least 6 contiguous subunits corresponding to any of the nucleotide sequences:

5' 3'
25 GGCTCCATTTCTTGCTCTCC
CATTTCCTTGCTCTCCTCTGT
GCTATGTCGACACCCAATTC
CCGCCCCCTCGCCTCTTGCCG
CGGGTCCCCTCGGGATTGGG
30 CACCTTCTTCTTCTATTCTT

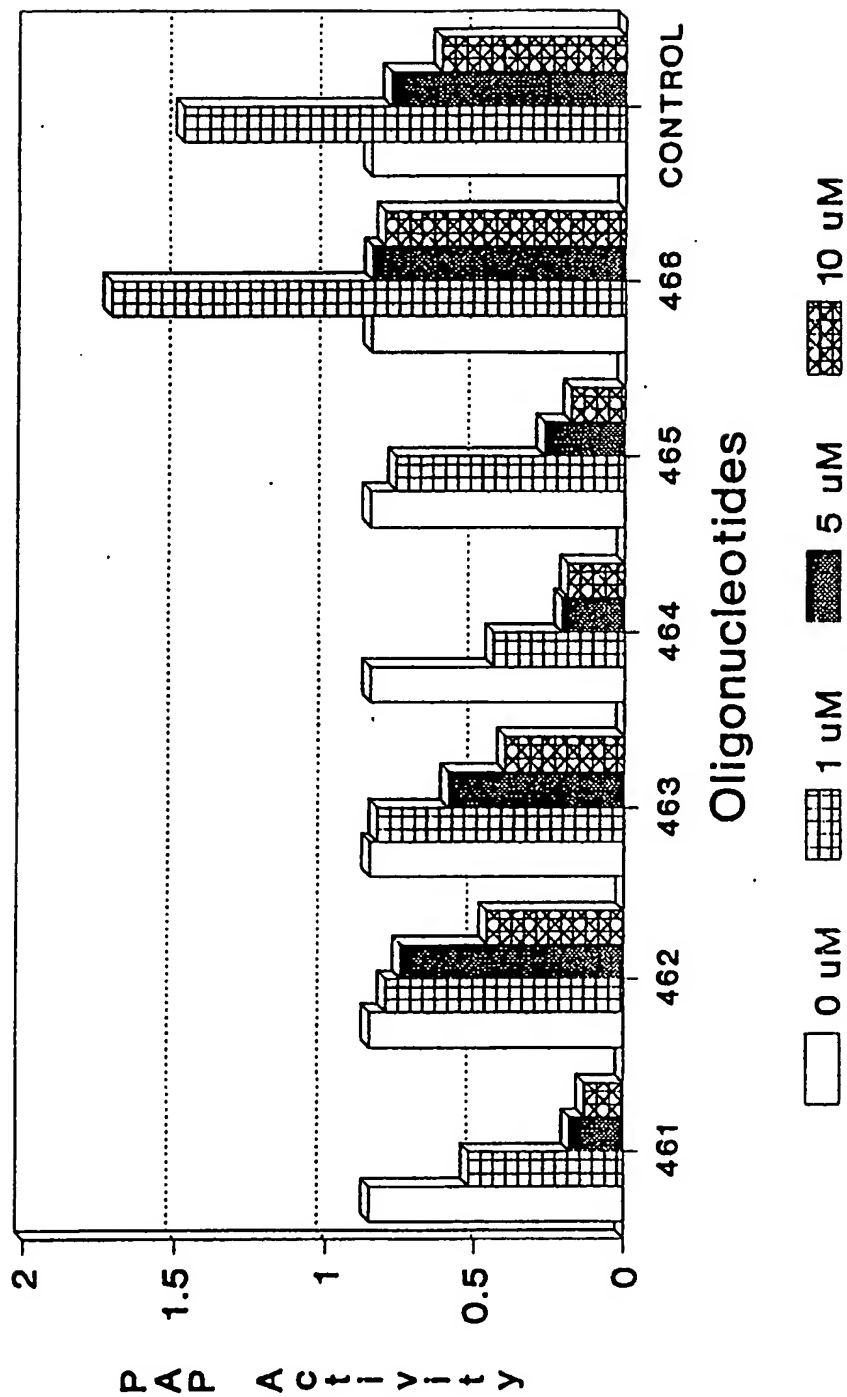
27. A method for modulating expression of the HIV tat gene comprising contacting cells or an animal infected with HIV with an oligonucleotide or oligonucleotide analog capable of binding with at least a portion of tat mRNA of HIV.

FIGURE 1

1 GGGUCUCUCU GGUUAGACCA GAUCUGAGCC UGGGAGCUCU CUGGCUAACU
51 AGGGAACCCA CUGCUAAGC CUCAAUAAAG CUUGCCUUGA GUGCUUCAAG
101 UAGUGUGUGC CCGUCUGUUG UGUGACUCUG GUAACUAGAG AUCCCUCAGA
151 CCCUUUAGU CAGUGUGGAA AAUCUCUAGC AGUGGCGCCC GAACAGGGAC
201 CUGAAAGCGA AAGGGAAACC AGAGCUCUCU CGACGCAGGA CUCGGCUUGC
251 UGAAGCGCCC GCACGGCAAG AGGCGAGGGG CGGCGACUGA AUUGGGUGUC
301 GACAUAGCAG AAUAGGCGUU ACUCGACAGA GGAGAGCAAG AAAUGGAGCC
351 AGUAGAUCCU AGACUAGAGC CCUGGAAGCA UCCAGGAAGU CAGCCUAAAA
401 CUGCUUGUAC CAAUUGCUAU UGUAAAAAGU GUUGCUUUA UUGCCAAGUU
451 UGUUUCAUAA CAAAAGCCUU AGGCAUCUCC UAUGGCAGGA AGAAGCGGAG
501 ACAGCGACGA AGACCUCCUC AAGGCAGUCA GACUCAUCAA GUUUCUCUAU
551 CAAAGCAACC CACCUCCTAA UCCCGAGGGG ACCCGACAGG CCCGAAGGAA
601 UAGAAGAAGA AGGUGGAGAG AGAGACAGAG ACAGAUCCAU UCGAUUAGUG
651 AACGGAUCCU UAGCACUUAU CUGGGACGAU CUGCGGAGCC UGUGCCUCUU
701 CAGCUACCAC CGCUUGAGAG ACUUAUCUU GAUUGUAACG AGGAUUGUGG
751 AACUUCUGGG ACGCAGGGGG UGGGAAGCCC UCAAUAUUG GUGGAAUCUC
801 CUACAGUAUU GGAGUCAGGA GCUAAAGAAU AGUGCUGUUA GCUUGCUCAA
851 UGCCACAGCU AUAGCAGUAG CUGAGGGGAC AGAUAGGGUU AUAGAAGUAG
901 UACAAGGAGC UUAUAGAGCU AUUCGCCACA UACCUAGAAG AAUAAGACAG
951 GGCUUGGAAA GGAUUUUGCU AUAAGAUGGG UGGCAAGUGG UCAAAAAGUA
1001 GUGUGGUUGG AUGGCCUGCU GUAAGGGAAA GAAUGAGACG AGCUGAGCCA
1051 GCAGCAGAUG GGGUGGGAGC AGCAUCUCGA GACCUAGAAA AACAUGGAGC
1101 AAUCACAAGU AGCAACACAG CAGCUAACAA UGCUGAUUGU GCCUGGCUAG
1151 AAGCACAAGA GGAGGAGGAG GUGGGUUUUC CAGUCACACC UCAGGUACCU
1201 UUAAGACCAA UGACUUACAA GGCAGCUGUA GAUCUUAGCC ACUUUUUAAA
1251 AGAAAAGGGG GGACUGGAAG GGCUAUUUA CUCCCAACGA AGACAAGUA

1301 UCCUUGAUCU GUGGAUCUAC CACACACAAG GCUACUUCCC UGAUUAGCAG
1351 AACUACACAC CAGGGCCAGG GAUCAGAUAU CCACUGACCU UUGGAUGGUG
1401 CUACAAGCUA GUACCAGUUG AGCCAGAGAA GUUAGAAGAA GCCAACAAAG
1451 GAGAGAACAC CAGCUUGUUA CACCCUGUGA GCCUGCAUGG AAUGGAUGAC
1501 CCGGAGAGAG AAGUGUUAGA GUGGAGGUUU GACAGCCGCC UAGCAUUUCA
1551 UCACAUGGCC CGAGAGCUGC AUCCGGAGUA CUUCAAGAAC UGCUGACAUC
1601 GAGCUUGCUA CAAGGGACUU UCCGCUGGGG ACUUUCCAGG GAGGCGUGGC
1651 CUGGGCGGGA CUGGGGAGUG GCGAGCCCUC AGAUCCUGCA UAUAAGCAGC
1701 UGCUUUUUGC CUGUACUGGG UCUCUCUGGU UAGACCAGAU CUGAGCCUGG
1751 GAGCUCUCUG GCUAACUAAG GAACCCACUG CUUAAGCCUC AAUAAAGCUU
1801 GCCUUGAGUG CUGUCAAAAA AAAAAAAAAA AAA

FIG 2



Standard Deviation 0 uM = 0.29

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/02734

I. CLASSIFICATION OF SUBJECT MATTER (In several classification symbols apply, indicate all) ⁵		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC (5th Ed.): C 07H 21/04; A 61 K 31/70		
US Cl.: 536/27, 536/28, 536/29; 514/44		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
US Cl.:	536/27, 536/28, 536/29; 514/44	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
Sequence Search: claimed sequences and complements thereto		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	PROC. NAT. ACAD. SCI USA, volume 85, issued October 1988, (WASHINGTON, D.C.), Agrawal et al., "Oligodeoxynucleoside phosphoramidates and phosphorothioates as inhibitors of human immunodeficiency virus," pages 7079-7083, see whole document.	1-27
Y	PROC. NAT. ACAD. SCI USA, volume 86, issued October 1989, (WASHINGTON, D.C.), Agrawal et al., "Inhibition of human immunodeficiency virus in early infected and chronically infected cells by antisense oligodeoxynucleotides and their phosphorothioate analogues," pages 7790-7794, see whole document.	1-27
<p>[*] Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>¹³ document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
05 August 1991		20 AUG 1991
International Searching Authority		Signature of Authorized Officer
ISA/US		L. Eric Crane

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	NATURE, Volume 334, Issued 14 July 1988, (LONDON, GB), Feng et al., "HIV-1 tat <i>trans</i> -activation requires the loop sequence with <i>tar</i> ," pages 165-167, see whole document.	1-27
Y	THE EMBO JOURNAL, Volume 8(3), Issued 1989, (LONDON, GB), Garcia et al., "Human immunodeficiency virus type 1 LTR TATA and TAR region sequences required for transcriptional regulation," pages 765-778, see whole document.	1-27
Y	PROC. NAT. ACAD. SCI USA, volume 85, issued August 1988, (WASHINGTON, D.C.), Goodchild et al., "Inhibition of human immunodeficiency virus replication by antisense oligodeoxynucleotides," pages 5507-5511, see whole document.	1-27
A	SCIENTIFIC AMERICAN, Issued October 1988, (U.S.A.), Haseltine et al., "The Molecular Biology of the AIDS Virus," pages 52-62, see whole document.	1-27
A	PROC. NAT. ACAD. SCI USA, volume 85, issued September 1988, (WASHINGTON, D.C.), Henthorn et al., "Expression of a human placental alkaline phosphatase gene in transfected cells: Use as a reporter for studies of gene expression," pages 6342-6346, see whole document.	1-27
Y	CELL, Volume 59, Issued 20 October 1989, (U.S.A.), Laspias et al., "HIV-1 Tat Protein Increases Transcriptional Initiation and Stabilizes Elongation," pages 283-292, see whole document.	1-27
Y	PROC. NAT. ACAD. SCI USA, volume 86, issued September 1989, (WASHINGTON, D.C.), Letsinger et al., "Cholesteryl-conjugated oligonucleotides: Synthesis, properties, and activity as inhibitors of replication of human immunodeficiency virus in cell culture," pages 6553-6556, see whole document.	1-27

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication where appropriate, of the relevant passages	Relevant to Claim No.
A	ANALYTICAL BIOCHEMISTRY, volume 172, Issued 1988, (U.S.A.), Marcus-Sekura, "Techniques for Using antisense Oligonucleotides to Study Gene Expression," pages 289-295, see whole document.	1-27
A	PROC. NAT. ACAD. SCI. USA, volume 84, issued November 1987, (WASHINGTON, D.C.), Matsukura et al., "Phosphorothioate analogs of oligodeoxynucleotides: Inhibitors of replication and cytopathic effects of human immunodeficiency virus," pages 7706-7710, see whole document.	1-27
A	NUCLEIC ACIDS RESEARCH, volume 17(20), issued 1989, (OXFORD, GB), Mori et al., "Phosphoroselenoate oligonucleotides: synthesis, physico-chemical characterization, anti-sense inhibitory properties and anti-HIV activity," pages 8207-8219, see whole document.	1-27
X	PROC. NAT. ACAD. SCI. USA, volume 85, issued October 1988, (WASHINGTON, D.C.), Sarin et al., "Inhibition of acquired immunodeficiency syndrome virus by oligodeoxynucleotide methylphosphonates," pages 7448-7451, see whole document.	1-27
Y	CELL, volume 59, Issued 20 October 1989, (U.S.A.), Sharp et al., "HIV TAR: an RNA Enhancer?" pages 229-230, see whole document.	1-27
	NUCLEIC ACIDS RESEARCH, Volume 17 (1), Issued 1989, (Oxford, GB), Shibahara et al., "Inhibition of human immunodeficiency virus (HIV-1) replication by synthetic oligo-RNA derivatives," pages 239-252, see whole document.	1-27
A	CANCER RESEARCH, Volume 48, issued 15 May 1988, (U.S.A.), Stein et al., "Oligodeoxynucleotides as Inhibitors of Gene Expression: A Review," pages 2659-2668, see whole document.	1-27

DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	J. GENERAL VIROLOGY, volume 70, Issued 1989, (GB), Stevenson et al., "Inhibition of Human Immunodeficiency Virus Type 1-mediated Cytopathic Effects by Poly(l-lysine)-conjugated Synthetic Antisense Oligonucleotides," pages 2673-2682, see whole document.	1-27
A	BIOTECHNIQUES, volume 6(10), issued 1988, van der Krol et al., "Modulation of Eukaryotic Gene Expression by Complementary RNA or DNA Sequences," pages 958-976, see whole document.	1-27
A	GENES & DEVELOPMENT, volume 2, issued 1988, Walder et al., "Antisense DNA and RNA: progress and prospects," pages 502-504, see whole document.	1-27
Y	JOURNAL OF VIROLOGY, volume 62(10), issued October 1988, (U.S.A.), Zaia et al., "Inhibition of Human Immunodeficiency Virus by Using an Oligonucleoside Methylphosphonate Targeted to the <i>tat-3</i> Gene," pages 3914-3917, see whole document.	1-27
A	PHARMACEUTICAL RESEARCH, volume 5(9), issued 1988, (New York, New York, U.S.A.), Zon, "Oligonucleotide Analogues as Potential Chemotherapeutic Agents," pages 539-549, see whole document.	1-27
Y	PROC. NAT. ACAD. SCI USA, volume 83, issued June 1988, (WASHINGTON, D.C.), Zamecnik et al., "Inhibition of replication and expression of human T-cell lymphotropic virus type III in cultured cells by exogenous synthetic oligonucleotides complementary to viral RNA," pages 4143-4146, see whole document.	1-27

DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Page(s) of Claim No.
A	JOURNAL OF PROTEIN CHEMISTRY, volume 6(2), issued 1987, (New York, New York, U.S.A.), Zon, "Synthesis of Backbone-Modified DNA Analogues for Biological Applications," pages 131-145, see whole document.	1-11
A	TIPS, Vol. 9, issued February 1988, "Antisense Nucleic Acids as a Potential Class of Pharmaceutical Agents", pages 45-47.	1-27

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